AD_____

Award Number: DAMD17-96-1-6212

TITLE: Training in Support of Research Project Entitled "Tumor Cell De-adhesion by Aberrant, Single Subunit Integrins"

PRINCIPAL INVESTIGATOR: Yan Zhang, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jul 96 - 30 Jun 00)

4. TITLE AND SUBTITLE
Training in Support of Research Project Entitled "Tumor Cell De-adhesion by Aberrant, Single Subunit Integrins"

5. FUNDING NUMBERS
DAMD17-96-1-6212

6. AUTHOR(S)
Yan Zhang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-MAIL:
yzhang@burnham-inst.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

I hypothesize that there are substantial differences in the plasma membrane complement of metastatic vs. non-metastatic tumor cells. To test my hypothesis, I prepared plasma membrane proteins from three breast carcinoma cell lines (MDA-MB-435, MDA-MB-231 and MCF-7) of differing degrees of invasiveness, and generated 2D polypeptide expression maps. Computer analysis of the 2D polypeptide patterns showed that the two non-metastatic cell lines (MCF-7 and MDA-MB-231) displayed 56% similarity in their proteins, whereas MCF-7 and the highly metastatic cell line, MDA-MB-435, displayed only 42% similarity in their proteins. Further analysis showed 81 polypeptide spots to be unique to the 2D gel of the MDA-MB-435 cell line. Five of these proteins were identified using techniques of mass spectrometry and database searching. As an additional method of identifying plasma membrane proteins with relevance to tumor progression, I applied the technique of antibody library phage display. I created scFv antibody libraries to the plasma membrane proteins of MCF-7 and MDA-MB-435 cells and enriched these libraries for antibodies to cell-surface proteins. By flow cytometry, I identified an individual phage clone that displays an antibody that recognizes a unique protein on the surface of the metastatic cells. The identification of proteins that are unique to metastatic cells will increase our knowledge of the molecular mechanisms that govern tumor progression.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

__X__ Where copyrighted material is quoted, permission has been obtained to use such material.

__X__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__X__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__X__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__N/A__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__N/A__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__N/A__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__N/A__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Yanzhang 7/26/2000

PI - Signature Date

TABLE OF CONTENTS

COVER	1
REPORT DOCUMENT PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	6-9
KEY RESEARCH ACCOMPLISHMENTS	10
REPORTABLE OUTCOMES	11
CONCLUSIONS	12
REFERENCES	13
BIBLIOGRAPHY	14

INTRODUCTION

This grant was originally awarded to Ian Jongewaard and was transferred to me. Along with the transfer of the award, I took a slightly different research direction. My revised statement of work was submitted and approved in December 1998.

I hypothesized that:

- there are substantial differences in the plasma membrane complement of metastatic vs. non-metastatic tumor cells
- there are substantial differences in the plasma membrane complement of adherent vs non-adherent tumor cells

My objectives were

- to use the techniques of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry to identify those plasma membrane proteins that were differentially expressed between metastatic and non-metastatic tumor cells and between adherent and non-adherent cells
- to use the technique of antibody phage display technology to identify cell surface proteins that differ in expression between the metastatic and non-metastatic cells.

The intention of my research was to gain a better understanding of the molecular mechanisms that govern metastasis. In addition, I hoped to identify new diagnostic markers or potential drug targets that might have significant value in treating breast cancer.

BODY

Here I describe my efforts as they relate to the revised Statement of Work which was approved when this research grant was transferred to me in December of 1998. As you will see, my emphasis has been on Aims Three and Four.

Aim One:
Compare the protein expression maps of tumor cells grown in suspension vs. tumor cells adherent to RGD ligand.

Task 1. Grow breast cancer cells in suspension and adherent to RGD ligand. Purify plasma membranes using colloidal silica and extract proteins using Urea.

Status: Not initiated

Task 2. Run two dimensional acrylamide gels to separate plasma membrane proteins. Set up the electrophoresis system such that at least 500 protein spots can be distinguished on all gels. Use computer software to assess similarities and differences among the two culture conditions.

Status: Not initiated

Aim Two:
Identify plasma membrane proteins that differ in expression level among adherent vs. non-adherent breast cancer cells.

Task 1. Extract from the 2D gels protein spots that differ between the two conditions and digest them with trypsin. Subject the digest to analysis with MALDI TOF mass spectrometry. Use these peptide mass signatures to identify the proteins of interest from computer databases (SwissProt etc.).

Status: Not initiated

Aim Three:
Generate two-dimensional maps of protein expression in metastatic and non-metastatic tumor cells.

Task 1. Grow non-metastatic and metastatic MDA-MB-435 breast cancer cells that have been isolated and characterized in this laboratory.

Status: Completed

Results: Three breast carcinoma cell lines with different metastatic potential were cultured. The cell lines were, MDA-MB-435, which is highly metastatic, and MCF-7 and MDA-MB-231, which are non-metastatic.

Task 2. Purify plasma membrane proteins from the two cell types, extract proteins and analyze the protein complement on 2D gels.

Status: Completed

Results:

- Two-dimensional protein expression maps were generated from the plasma membrane proteins of the three breast cancer cell lines. On average, 376 polypeptide spots were resolved on each of the 2D gels.

- The 2D gels were analyzed using Image Master software (Amersham Pharmacia Biotech). Calculation of % similarity in plasma membrane protein expression showed that 56% of the polpeptide spots matched between MCF-7 and MDA-MB-231 (two non-metastatic breast cancer cell lines), whereas only 42% of the polypeptide spots were the same between MCF-7 and MDA-MB-435 (metastatic vs non-metastatic breast cancer cell lines).

- In order to characterize the differential expression of protein between the metastatic and non-metastatic cells, lists and gel images were generated to describe all polypeptide spots that were present in the gel of the metastatic cell line and not in the non-metastatic cell lines and visa versa. Eighty-one polypeptide spots were found to be unique to the 2D gel of the metastatic cell line, and eighty polypeptide spots were found to be present in the 2D gels of the non-metastatic cell lines, yet absent in that of the metastatic cell line.

Limitations: While analyzing the 2D gels using the commercially available software, it became evident that considerable operator participation was necessary to ensure proper selection of individual spots and proper spot-matching between gels. Infact, I've often seen it stated in the literature that operator verification occurred during computer analysis of 2D-gel spot patterns. The spot-matching and subsequent analysis could have been completed more quickly had the software been capable of independent analysis.

Aim Four

Identify plasma membrane proteins that differ in expression between metastatic and non-metastatic breast cancer cells.

Task 1. Extract from the 2D gels protein spots that differ between the two conditions and digest them with trypsin. Subject the digest to analysis with MALDI TOF mass spectrometry. Use these peptide mass signatures to identify the proteins of interest from computer databases.

Status: Completed

Results:

- Numerous polypeptide spots, differentially expressed between the metastatic and non-metastatic cell lines, have been excised from the 2D gels and frozen in preparation for identification.

- Five proteins that are unique to the 2D gel of the metastatic cell line have been identified. These proteins are:

  Galectin-1
  Eukaryotic translation initiation factor 3—subunit 5 (eIF3-p47)
  alpha synuclein
  alpha enolase
  AAD27784 (Sptremlnew) uncharacterized gene

I am encouraged by my results because one of the proteins that I have identified, galectin-1, has previously been cited in the literature as having a potential role in tumor progression (1-3). In addition, several other proteins that I have identified, eIF3-p47 and alpha synuclein, have close family members that have been cited as being involved in tumor progression (4-8). This suggests that the proteins that I have identified should also be investigated for a potential role in metastasis. The fact that I have identified a protein (AAD27784) that has not yet been characterized, and whose function has not been reported in the literature, has potentially exciting consequences.

Limitations: Unfortunately, a large number of the differentially expressed polypeptide spots are feared to be of too low an abundance (lightly silver-stained) to be identifiable by our current MALDI-TOF mass spectrometry methods. However, a mass spectrometer with increased sensitivity will be introduced to the Burnham Institute within the next couple of months, allowing for possible identification of these proteins in the near future.

Note: Aim Four had been expanded (see annual summary report covering July 1 1998--June 30 1999) to include an additional methodology, antibody library phage display, for identifying the plasma membrane proteins that differ in expression between metastatic and non-metastatic breast cancer cells. The inclusion of this technique is of great importance in characterizing this difference in plasma membrane protein expression because it became apparent during my study that current 2D gel technology, despite improvements in protein solubility, is still inadequate at representing hydrophobic transmembrane proteins.

Status: Initiated

Results:

- Using techniques similar to those described in the literature (9-12), I have constructed phage display single chain variable fragment (scFv) antibody libraries to the plasma membrane proteins of MCF-7 cells (non-metastatic) and MDA-MB-435 cells (highly metastatic). The number of independent transformants are 5.7×10^9 for the library against MCF-7 cells and 2.7×10^9 for the library against MDA-MB-435 cells. These values ensure that the representation of pairs of variable fragment antibodies against numerous antigens is very high and the chance of obtaining high affinity antibodies is also increased.

- Using antibodies against the Flag epitope and the pIII protein, I have confirmed by Western Blot that the fusion protein (pIII-scFv-Flag, ~100kD) is present on the surface of the phage.

- Using intact cell-based panning methods described in the literature (17), I have selected for subsets of scFv antibodies that recognize antigens on the surface of MCF-7 cells and MDA-MB-435 cells. Flow cytometry studies confirmed that these modified phage display scFv antibody libraries (enriched for antibodies to cell-surface proteins) are displaying antibodies that are capable of binding to proteins at the surface of the corresponding cell line.

- I have used flow cytometry to evaluate individual scFv phages from these cell-surface scFv antibody libraries in order to identify those that are displaying antibodies to unique proteins of either cell line. Although I have just begun this effort, I have already identified one scFv antibody that is unique to the metastatic MDA-MB-435 cells. I have sequenced the scFv antibody code sequence, and I am in the process of subcloning and expressing a soluble form of this scFv antibody which will be used for identifying the corresponding cell surface protein.

KEY RESEARCH ACCOMPLISHMENTS

- I generated two-dimensional polypeptide expression maps from the plasma membrane proteins of three breast cancer cell lines of differing degrees of invasiveness.

- Through analysis of the two-dimensional polypeptide expression maps, I demonstrated that the two non-metastatic cell lines (MCF-7 and MDA-MB-231) displayed 56% similarity in their proteins, whereas MCF-7 and the highly metastatic cell line, MDA-MB-435, displayed 42% similarity in their proteins.

- I generated a list and gel image describing eighty-one polypeptide spots that were unique to the 2D gel of the MDA-MB-435 cells.

- I generated lists and gel images describing eighty polypeptide spots that were present in the 2D gels of the non-metastatic cell lines, yet absent in that of the metastatic cell line.

- I excised and froze numerous gel pieces containing polypeptides that showed differential expression between metastatic and non-metastatic cell lines.

- Using mass spectrometry, I identified five proteins that were uniquely present in the 2D gel of the metastatic cell line.

- I created phage display scFv antibody libraries of high complexity to the plasma membrane proteins of MCF-7 and MDA-MB-435 cells.

- I demonstrated by Western Blot that the fusion protein (pIII-scFv-Flag, ~100kD) is present on the surface of the phage.

- Using intact cell-panning methods, I successfully enriched my scFv antibody library for phage clones displaying antibody to cell surface proteins of MCF-7 and MDA-MB-435 cells.

- By flow cytometry, I identified an individual phage clone that is displaying a scFv antibody that recognizes an antigen unique to the surface of the metastatic breast carcinoma cell line, MDA-MB-435.

REPORTABLE OUTCOMES

- Manuscript:
  Harvey, S., Zhang, Y., Miller, C., and Smith, J.W. Charting the Plasma Membrane Signature of Invasive Breast Cancer. (In preparation)

  Note: upon completion of the above mentioned manuscript, copies will be forwarded to the USAMRMC.

CONCLUSIONS

The objectives of my study were:

- to use the techniques of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry to identify those plasma membrane proteins that were differentially expressed between metastatic and non-metastatic tumor cells.
- to use the technique of antibody phage display technology to identify cell surface proteins that differ in expression between the metastatic and non-metastatic cells.

I believe that I was successful in my pursuit of these objectives. The 2D-PAGE allowed me to visualize the differential polypeptide expression pattern between the metastatic (MDA-MB-435) and non-metastatic (MDA-MB-231 and MCF-7) breast cancer cell lines, and MALDI-TOF mass spectrometry allowed me to identify some of the proteins that were either common to all cell lines or that were unique to the metastatic cell line. One limitation that I encountered during the protein identification phase of my work was that many of the polypeptide spots were very low in abundance and could not be identified using our MALDI-TOF mass spectrometer instrument. Fortunately, a nanoelectrospray mass spectrometer has recently been acquired which should help with additional protein identifications. The identification of the proteins that are potentially relevant to tumor progression has important implications for increasing the understanding of the molecular mechanisms that govern metastasis. The fact that my approach addresses global protein changes rather than that of just a single protein, is of particular importance in increasing the understanding of such a multifactorial process as cancer progression.

While performing MALDI-TOF mass spectrometry on a variety of proteins from the 2D gels, it became evident that a large percentage of the proteins represented on the gel were plasma membrane associated proteins (either at the cell surface or at the cytoplasmic side) and not membrane-spanning proteins. Transmembrane proteins, containing multiple hydrophobic domains, are more difficult to solubilize in the water-based buffers required for 2D electrophoresis. Although the composition of my solubilization buffer included the most recent advances towards protein solubility, the representation of highly hydrophobic proteins on 2D gels continues to be limited.

As an additional methodology for identifying cell-surface proteins (including transmembrane proteins) that differ in expression between the metastatic and non-metastatic cells I used the technique of antibody phage display. Although this effort has just begun, I have already identified an individual phage clone which expresses a scFv antibody to a protein that is unique to the metastatic cell line. This finding has important diagnostic and therapeutic implications. These scFv antibodies are, in essence, probes that can be used to identify disease-related cell surface markers, or as therapeutic targets.

REFERENCES

1. Perillo, N.L., Marcus M.E. and Baum L.G. 1998. Galectins:versatile modulators of cell adhesion, cell proliferation, and cell death. *J. Mol. Med.* 76:402-412.
2. Yamaoka, K., Mishima, K., Nagashima, Y., Asai, A., Sanai, Y. and Kirina, T. 2000. Expression of galectin-1 mRNA correlates with the malignant potential of human gliomas and expression of antisense galectin-1 inhibits the growth of 9 glioma cells. *J. Neurosci. Res.* 59:722-730.
3. Xu, X.C., el-Naggar, A.K. and Lotan, R. 1995. Differential expression of galectin-1 and galectin-3 in thyroid tumors. Potential diagnostic implications. *Am. J. Pathol.* 147:815-822.
4. Nupponen, N.N., Porkka, K., Kakkola, L., Tanner, M., Persson, K., Borg, A., Isola, J.and Visakorpi,T. 1999. Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. *Am. J. Pathol.* 154:1777-1783.
5. De Benedetti, A. and Harris, A.L. 1999. eIF3E expression in tumors: its possible role in progression of malignancies. *Int. J. Biochem. Cell Biol.* 31:59-72.
6. Jia, T., Liu, Y.E., Liu, J., and Shi, Y.E. 1999. Stimulation of breast cancer invasion and metastasis by synuclein γ. *Cancer Res.* 59:742-747.
7. Bruening, W., Giasson, B.I., Klein-Szanto, A.J., Lee, V.M., Trojanowski J.Q. and Godwin A.K. 2000. Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. *Cancer.* 88:2154-2163.
8. Clayton, D.F., and George, J.M. 1998. The synucleins:a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci.* 21:249-254.
9. Barbas, C.F., Bain, J.D., Hoekstra, D.M. and Lerner R.A. 1992. Semisynthetic combinatorial antibody libraries: A chemical solution to the diversity problem. *Proc. Natl. Acad. Sci. USA.* 89:4457-4461.
10. Barbas, C.F., Languina L.R. and Smith, J.W. 1993. High-affinity self-reactive human antibodies by design and selection:Targeting the integrin ligand binding site. *Proc. Natl. Acad. Sci.* USA. 90:10003-10007.
11. Barbas, C.F., Kang, A.S., Lerner, R.A. and Benkovic, S.J. 1991. Assembly of combinatorial antibody libraries on phage surfaces: The gene III site. *Proc. Natl. Acad. Sci. USA.* 88:7978-7982.
12. Williamson, R.A., Burioni, R., Sanna, P.P., Partridge, L.J., Barbas, C.F. and Burton, D.R. 1993. Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. *Proc. Natl. Acad. Sci. USA.* 90:4141-4145.
13. Noronha, E.J., Wang, X., Desai, S.A., Kageshita, T. and Ferrone, S. 1998. Limited diversity of human scFv fragments isolated by panning a synthetic phage-display scFv library with cultured human melanoma cells. *J. Immunol.* 161:2968-2976.

BIBLIOGRAPHY

Meeting Abstracts: None

Publications:

- Harvey, S., Zhang, Y., Miller, C., and Smith, J.W. Charting the Plasma Membrane Signature of Invasive Breast Cancer. (In preparation, 2000).

Personnel:

- Ian N. Jongewaard, Ph.D.
- Yan Zhang, Ph.D.